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Interspecific hybridization and gene flow of ALS resistance in *Amaranthus* species

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Several inbred lines of acetolactate synthase (ALS)-inhibiting herbicide-resistant (ALS-R) Palmer amaranth and ALS-susceptible (ALS-S) common waterhemp were developed in the greenhouse. Interspecific hybrids were obtained by allowing several ALS-S common waterhemp females to be pollinated only by ALS-R Palmer amaranth in a growth chamber. Putative hybrid progeny were treated with an ALS-inhibiting herbicide, and the hybrid nature verified using a polymorphism found in the parental ALS gene. Polymerase chain reaction (PCR) was used to amplify a region of the ALS gene in both parental plants and putative hybrids. Restriction enzyme digestion of the ALS-R Palmer amaranth PCR fragment resulted in two smaller fragments, whereas the PCR fragment in the ALS-S common waterhemp was not cut. Restriction digestion of the putative hybrid PCR fragment showed a combination of ALS-R Palmer amaranth double fragments and an ALS-S common waterhemp single fragment. Approximately 4 million flowers were present on 11 common waterhemp females and produced about 44,000 seeds that appeared viable. From the approximately 3,500 putative hybrid seedlings that were screened, 35 were confirmed as hybrids using herbicide resistance as a phenotypic and molecular marker. The data collected here verify that interspecific hybridization does occur between these two species, and even at a low rate, it could contribute to the rapid spread of ALS resistance in these species.

Nomenclature: Imazethapyr; common waterhemp, *Amaranthus rudis* Sauer AMATA; Palmer amaranth, *Amaranthus palmeri* S. Wats AMAPA.

Key words: Molecular markers, polymerase chain reaction, restriction endonuclease.

Amaranthus species, often collectively called pigweeds, are among the most troublesome agricultural weeds in the midwestern United States (Wax 1995). Their aggressive growth habit and allelopathic chemicals allow these plants to compete strongly with other plants (Barkely 1986; Keeley et al. 1987; Knezevic et al. 1997; Menges 1987, 1988; Murphy et al. 1996; Wax 1995). They reduce grain yield and quality and also hinder mechanical harvest of cultivated crops (Keeley et al. 1987; Knezevic et al. 1997; Murphy et al. 1996; Wax 1995). Reduced yields as a result of competition with *Amaranthus* species have been observed in several crops: 50% in corn (*Zea mays* L.), 68% in soybean [*Glycine max* (L.) Merr.], and 70% in cotton, *Gossypium hirsutum* (L.). (Gossett et al. 1992; Klingaman and Oliver 1994; Vizantinopoulos and Katranis 1998). In addition, some *Amaranthus* species have been shown to possess compounds that may induce toxicosis and death in livestock (Kerr and Kelch 1998).

Infestations of field crops with several species of *Amaranthus* have increased rapidly in the last several years. Increased awareness of environmental issues has led to the adoption of reduced tillage and a greater reliance on environmentally safe herbicides. These changes in cultural practices have led to shifts in weed populations to biotypes that are better suited to their environment (Wax 1995).

At least 10 *Amaranthus* species are prevalent in the midwestern United States (Horak et al. 1994; Wax 1995). Two of the more prominent species are common waterhemp (*Amaranthus rudis*) and Palmer amaranth (*Amaranthus palmeri*). Both of these native species commonly are found in disturbed agroecosystems. Common waterhemp occurs mainly in the eastern half of the United States, whereas Palmer amaranth occurs mainly in the southern half. Mixed populations of these two species are common where their distributions overlap. Both species are dioecious and, therefore, obligate cross-pollinators, which raises the issue of potential hybridization between them. These interspecific hybrids may not be easily distinguishable because they may exhibit characteristics of either or both parents (Barkely 1986; Horak et al. 1994).

Acetolactate synthase (ALS)-inhibiting herbicides (acetohydroxyacid acid [AHAS]; EC 4.1.3.18) are used widely to control grass and broadleaf weeds (Beyer et al. 1988). Several herbicide families, including imidazolinone, sulfonylurea, triazolpyrimidines, and pyrimidinyl oxybenzoate effectively inhibit the ALS enzyme (Saari et al. 1994).

The ALS-inhibiting herbicides were accepted widely for their low cost, minimal mammalian toxicity, broad-spectrum control, and high efficacy (Saari et al. 1994). As a result, they were applied extensively for the control of troublesome weeds, including the *Amaranthus* species. In 1987, 5 yr after their introduction, the first case of resistance to an ALS inhibitor was reported in prickly lettuce (*Lactuca serriola* L.) (Mallory-Smith et al. 1990). Since then, 64 species have evolved resistance to the ALS inhibitors (Heap 2000).

In 1995 in Kansas, Palmer amaranth and common waterhemp biotypes were confirmed to have resistance to ALS

inhibitors (Horak and Peterson 1995). These two biotypes were characterized as having cross-resistance to imazethapyr and thifensulfuron, both ALS enzyme inhibitors. In vitro examination of the ALS-resistant biotypes of Palmer amaranth and common waterhemp biotypes determined that resistance was due to an insensitive enzyme (Sprague et al. 1997). Other studies have shown that a single nucleotide substitution in the ALS gene can confer resistance to an ALS inhibitor (Guttieri et al. 1992; Woodsworth et al. 1996).

It is unknown whether the resistant biotypes of Palmer amaranth and common waterhemp developed resistance independently or whether one species developed the resistance and transferred it to the other via interspecific hybridization. This research was undertaken to assess the extent to which the latter scenario might occur. The objective of this study was to assess the potential for interspecific hybridization between the two weedy species common waterhemp and Palmer amaranth using ALS resistance as a phenotypic and molecular marker to identify interspecific hybrids.

Materials and Methods

Plant materials. Several inbred lines of ALS-resistant (ALS-R) Palmer amaranth and ALS-susceptible (ALS-S) common waterhemp were developed to ensure that these two species were breeding true for their respective response to the ALS-inhibiting class of herbicides. Because of the dioecious nature of each species, inbred lines were produced utilizing a full-sib mating scheme (Figure 1) (Poehlman and Sleper 1995). Five to 10 ALS-R Palmer amaranth and ALS-S common waterhemp seedlings were grown in 5-cm-diam conical pots filled with 100 g of commercial potting mixture.[1] Plants were thinned to one per pot after emergence. The potting mixture was steamed for 1.0 h to reduce the risk of contaminant seed. Greenhouse temperatures were 30/25 ± 3 C (day/night) with a 16:8 h (day : night) photoperiod. Supplemental light intensity was 80 μmol m^{-2} s^{-1} photosynthetic photon flux (PPF). Plants were watered as needed and fertilized weekly with a commercial fertilizer[2] containing 300 μg L^{-1} nitrogen, 250 μg L^{-1} phosphorus, and 220 μg L^{-1} potassium.

At the two- to four-leaf stage, ALS-R Palmer amaranth seedlings were treated with imazethapyr[3] applied at 560 g ha^{-1} with 0.25% (v/v) nonionic surfactant[4] using a Research Track Sprayer[5] equipped with an 80015LP[6] tip and calibrated to deliver 187 L ha^{-1}. A random selection of ALS-S common waterhemp plants was sprayed to verify susceptibility. Visual evaluations of injury were taken 2 wk after treatment (WAT). Palmer amaranth plants exhibiting any imazethapyr injury symptoms were discarded.

Immediately following herbicide application, ALS-R Palmer amaranth and ALS-S common waterhemp were placed in separate growth chambers. The growing conditions were 30/25 ± 3 C (day/night), 70 ± 5% relative humidity, a 16:8 h (day : night) photoperiod, and 350 μmol m^{-2} s^{-1} PPF. When plants reached 25 to 30 cm in height, the growth chamber photoperiod was changed to 8:16 h (day : night) to induce flower initiation. The photoperiod was returned to the initial settings once flowering had been initiated. Plant sex was determined, and the inflorescences were covered with pollination bags to reduce uncontrolled crossing. When male and female plants were flowering simultaneously, one male and one female inflorescence were placed together in a pollination bag. Plants were shaken every morning during pollen anthesis to ensure even pollen dispersion. ALS-R Palmer amaranth × ALS-R Palmer amaranth and ALS-S common waterhemp × ALS-S common waterhemp crosses were performed by this method with each species in separate chambers. At maturity, the female inflorescence was clipped from the plant, and the seeds were removed and labeled with the parental identifications. Cleaned seeds were stored at room temperature for 3 wk to break dormancy.

Full-sib mating. Seedlings of ALS-R Palmer amaranth × ALS-R Palmer amaranth and ALS-S common waterhemp × ALS-S common waterhemp cross were grown in the greenhouse as described above. At the four- to six-leaf stage, ALS-R Palmer amaranth plants were treated with imazethapyr as described above, and those exhibiting imazethapyr injury symptoms were discarded. Plants from each species were moved to separate growth chambers and grown as described above. First generation full-sib seed was created by mating pairs of plants within the progeny of a single female plant. Full-sib crosses were conducted as described above. First generation full-sib seed was harvested, cleaned, and labeled. Second and third generation full-sib seed was obtained by repeating the above procedure for an additional two generations. Three generations of full-sib mating should result in inbred lines that breed true for their respective responses to the ALS-inhibiting herbicide.

Interspecific hybridization. Parental lines (third generation full-sib seeds) of ALS-R Palmer amaranth and ALS-S common waterhemp were grown in the greenhouse as described above for the full-sib lines. At the four- to six-leaf stage, the two newest leaves were harvested for subsequent DNA extraction from all potential parents for each species. Both species were allowed to grow 25 to 30 cm tall and moved to separate growth chambers with an 8:16 h (day : night) photoperiod. Plants were monitored for flower initiation. When the sex of the ALS-S common waterhemp could be determined, the female inflorescences were covered with a pollination bag, and the male ALS-S common waterhemp plants were removed from the chamber. Following flower initiation, growth chambers were returned to the 16:8 h (day : night) photoperiod. Interspecific hybrid crosses were performed by placing male ALS-R Palmer amaranth plants in a chamber with female ALS-S common waterhemp plants. Pollination bags were removed from the female ALS-S common waterhemp inflorescences. Female ALS-S common waterhemp flowers were receptive to pollen approximately 1 to 3 d prior to pollen dispersion. One week following initial pollen release from the ALS-R Palmer amaranth plants, additional male ALS-R Palmer amaranth were introduced to the chamber to extend the effective pollination duration.

Flower counting and seed harvest. Three weeks prior to harvest, the approximate number of flowers was determined for each female ALS-S common waterhemp plant. Because flower density on inflorescences was not consistent for the entire plant, each plant was divided into three sections: upper, middle, and lower. A single 5-cm inflorescence sample was selected from each section, and the flowers were counted using a dissecting microscope at 2× magnification.

When the plants reached maturity, the inflorescence was

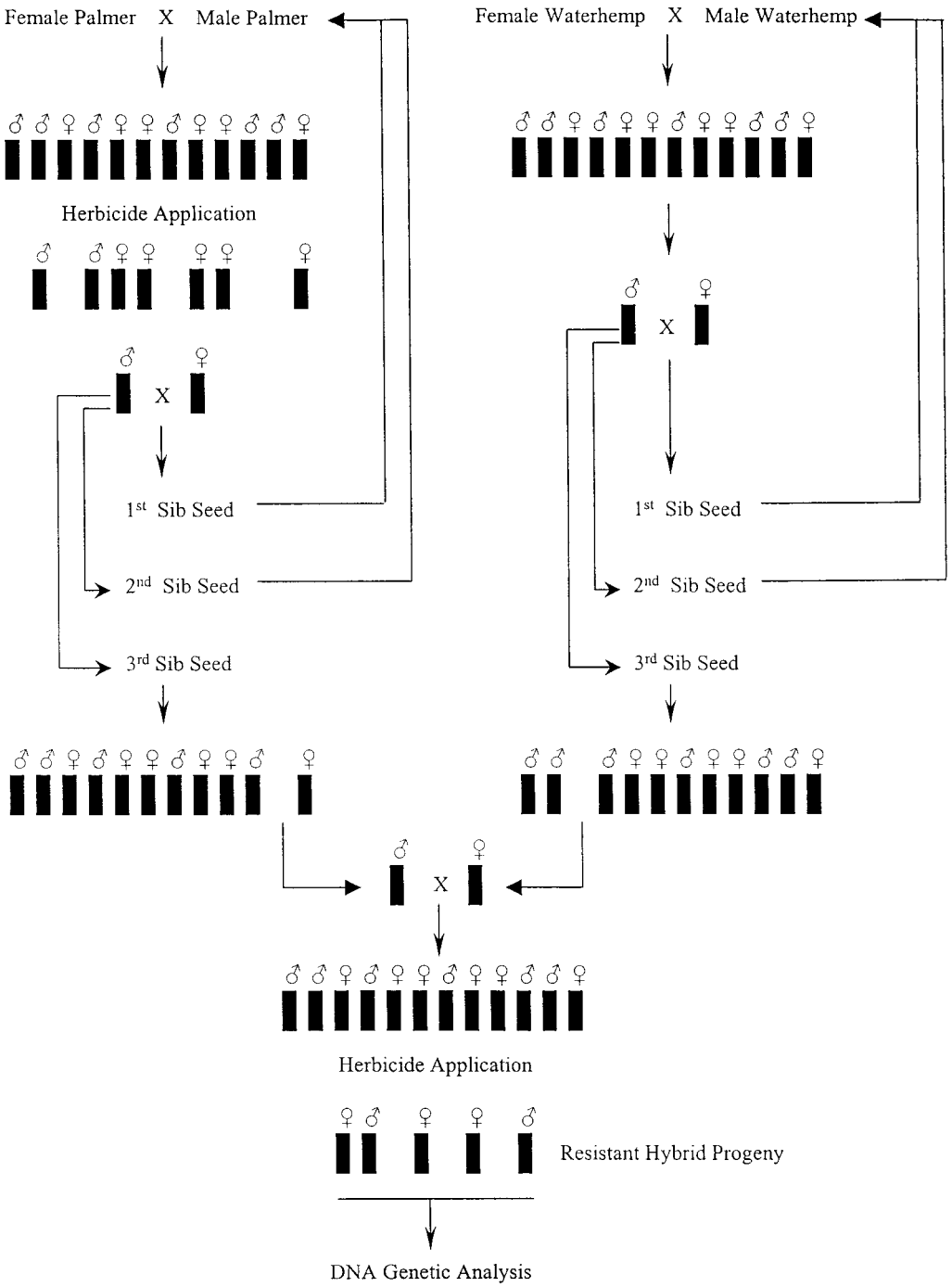

FIGURE 1. Diagram outlining the production of inbred lines of acetolactate synthase (ALS)-resistant Palmer amaranth (*Amaranthus palmeri*) and ALS-susceptible common waterhemp (*Amaranthus rudis*) and interspecific hybrids.

removed from the main stem, and the linear length for each section was measured. The inflorescence length of each section was multiplied by its corresponding flowers per centimeter count to estimate the number of flowers potentially crossed on each plant.

Seeds were threshed from each female common waterhemp and kept separate. The number of seeds per plant was determined by dividing the weight of the seed per plant by the weight of 250 seeds and multiplying by 250.

Hybrid screening. Putative hybrid seed were sown into 35- by 51-cm flats filled with commercial potting soil in the greenhouse under the conditions described above. At the four- to six-leaf stage, the two newest leaves were sampled from a random selection of seedlings for subsequent DNA analysis. Seedlings then were treated with 140 g ha^{-1} imazethapyr as described above. Visible injury was assessed as described earlier, and the susceptible plants were counted and discarded. The remaining plants were treated with 280 g ha^{-1} imazethapyr as described above to ensure imazethapyr resistance. Leaf tissue was collected from surviving plants for subsequent DNA analysis.

DNA extraction and analysis. Fresh leaf tissue was placed in a 1.5-ml centrifuge tube and frozen with liquid nitrogen. Frozen tissue was ground to a fine powder with a blunt-end

1-ml pipette tip and immediately suspended in an extraction buffer containing 100 mM Tris (pH 8.0), 50 mM EDTA, 500 mM NaCl, and 10 mM β-mercaptoethanol. The DNA was extracted as described by Dellaporta et al. (1983), resuspended in a storage buffer containing 10 mM Tris·Cl (pH 7.0) and 1 mM EDTA, quantified[7] at a wavelength of 260 nm, and stored at 4 C.

Forward and reverse nondegenerate oligonucleotides (primers) were synthesized[8] using known sequences of the ALS gene of *Amaranthus* species (Wetzel et al. 1999). The primers targeted a portion of the ALS gene that has been reported to contain mutations that confer broad range resistance to the ALS inhibitors in *Amaranthus* species (Woodsworth et al. 1996). The polymerase chain reaction (PCR) primer sequences were forward: 5′-GGG GAC GCA ATT CCT CCG C-3′ and reverse: 5′-GCC CTT CTT CCA TCA CCC TCT GTG-3′. Each PCR reaction was carried out in a 50 μl volume consisting of 250 ng genomic DNA, 3.5 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, 67 mM Tris (pH 8.8), 0.2 mM each deoxynucleotide triphosphate (dNTP), 0.4 mM of each primer, and 0.5 units thermostable DNA polymerase.[9] Duplicate reactions were performed for each plant sample in a heated-bonnet thermal cycler[10] programmed for an initial denaturation temperature of 94 C for 5 min followed by 25 cycles of 20 s at 94 C, 1 min at 62 C, and 1 min at 72 C. After the final cycle, the block was cooled and held at 4 C until analysis. A 5 μl aliquot of the PCR product was resolved on a 1.0% TBE agarose[11] gel containing 45 mM Tris (pH 8.0), 1 mM EDTA, and 0.1 μg ml^{-1} ethidium bromide at 50 volts for 1 hr. A 100-base pair (bp) mass ladder[12] was used as a reference. The gel was viewed and photographed on an ultraviolet light box to confirm product amplification.

DNA sequencing. Following successful PCR amplification, unincorporated primers and dNTPs were removed by resolving a 30-μl aliquot of the PCR reaction on a 1% modified TAE agarose gel containing 40 mM Tris (pH 8.0) and 0.1 mM EDTA. The PCR-amplified fragment was viewed using ultraviolet light and was excised from the gel. The excised band was placed in a centrifuge filter apparatus[13] and centrifuged at 5,000 × *g* for 10 min. The purified product was quantified using a spectrophotometer at 260 nm wavelength. The PCR fragment was precipitated with 0.3 M sodium acetate and 70% ethanol and centrifuged at 22,000 × *g* for 20 min. The supernatant was removed, and the pellet resuspended in a volume of distilled water that would result in a DNA concentration of 15 ng μl^{-1}. A commercially available sequencing kit[14] was used for subsequent sequence analysis. Sequencing reactions were conducted in a 20-μl volume consisting of 30 ng PCR product, 0.25 pmol forward primer, and 8.0 μl Terminator Ready Reaction Mix. Reactions were performed in a heated-bonnet thermal cycler using the same program as described for PCR amplification. The final products of the sequence reaction were sent to the KSU-USDA[15] Sequencing Facility for sequence reading. Sequences were viewed and analyzed using genetic analysis software.[16]

Restriction enzyme digestion. The differences found in the ALS gene sequences of ALS-R Palmer amaranth and ALS-S common waterhemp allowed the use of the Mfe1 restriction endonuclease enzyme[17] to differentiate between the two biotypes. Restriction digests were carried out in a 30-μl volume consisting of 10 μl PCR product, 5 mM potassium acetate, 2 mM Tris (pH 7.9), 1 mM magnesium acetate, 0.1 mM dithiothereitol, and 0.5 units Mfe1 restriction enzyme. Reactions were incubated in a heated-bonnet thermal cycler at 37 C for 3 h. Digested products were run on a 1.5% TBE gel at 80 volts for 1 h, and each lane of digested product was loaded next to a corresponding lane of undigested product for a reference. Banding patterns were visualized using ultraviolet light, and each sample was scored using a + (cleaved)/− (uncleaved) scoring system.

Cloning and sequencing. In order to verify the resulting banding patterns associated with the hybrid progeny, the uncut fragment was cloned and subsequently sequenced. Following restriction digestion of the hybrid progeny, the intact band associated with the susceptible common waterhemp was excised from a gel and purified as described above. The purified fragment was cloned using a commercially available PCR fragment cloning kit.[17] A 10 μl ligation reaction was performed in a ligation buffer containing 30 mM Tris (pH 7.8), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM adenosine triphosphate, 5% polyethylene glycol, 15 ng PCR product, 3 Weiss units T4 DNA ligase, and 25 ng linearized plasmid. The ligation reactions were incubated for 12 h at 4 C and transformed into commercially available competent cells[18] following the manufacturer's instructions. Transformed cells were plated onto Luria–Bertani (LB) media[19] (pH 7.0) culture plates supplemented with 100 μg ml^{-1} ampicillin, 80 μg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactoside, and 0.5 mM isopropylthio-β-D-galactoside. Plates were incubated for 16 to 24 h at 37 C.

Plasmid extraction and sequencing. Several putative PCR fragment clones were selected as white bacterial colonies and grown in liquid nutrient media supplemented with 100 μg ml^{-1} ampicillin. Cultures were incubated for 12 h on a rotary table set at 150 rpm and 37 C. Plasmid was extracted using a commercially available miniprep plasmid extraction kit,[20] and the recovered plasmid was sent to the KSU-USDA Sequencing Facility for sequencing.

Experimental design and data analysis. The ALS-S common waterhemp and ALS-R Palmer amaranth were planted in the greenhouse and random crosses were made within each species to develop inbred progeny lines. The potential for interspecific hybridization was assessed using a random selection of 11 inbred ALS-S common waterhemp female plants crossed with five ALS-R Palmer amaranth male plants. Plants were arranged in the growth chamber in a completely randomized design. The mean numbers of flowers present, seeds produced, and seedlings screened for each plant were determined from three repeated measurements from each plant. Means were separated using standard errors.

Results and Discussion

Flower counts. Approximately 4 million flowers were present on 11 ALS-S common waterhemp plants that potentially could have produced interspecific hybrid progeny. Counts for individual plants ranged from 132,000 to 658,000 flowers (Table 1). However, only a small percentage of these flowers produced apparently normal seed. Seed production from the flowers ranged from 0.02 to 4.03%; a large amount of "aborted," or flat seed, was produced and was

TABLE 1. Total numbers of flowers and seeds set and percent seed set for each of the 11 ALS-S common waterhemp (*Amaranthus rudis* AMATA) plants used to produce interspecific hybrids.[a,b]

Female AMATA plant	Flowers	Seed set	% seed set
	Flowers/plant	Seeds/plant	%
1	397,500 ± 31,000	14,300 ± 1,500	3.60 ± 1.70
2	216,000 ± 22,000	900 ± 100	0.40 ± 0.50
3	658,000 ± 87,000	3,200 ± 300	0.50 ± 0.30
4	270,700 ± 20,000	5,000 ± 200	1.90 ± 0.80
5	406,000 ± 24,000	2,000 ± 300	0.50 ± 0.10
6[c]	140,000 ± 17,000	5,600 ± 20	4.00 ± 1.90
7	498,000 ± 79,000	8,700 ± 2,200	1.80 ± 1.20
8	550,000 ± 24,000	1,200 ± 100	0.20 ± 0.10
9	133,000 ± 4,000	660 ± 70	0.50 ± 0.20
10	338,000 ± 23,000	2,300 ± 100	0.70 ± 0.10
11[d]	354,000 ± 8,000	55 ± 4	0.02 ± 0.002
Total	3,959,000 ± 14,000	43,800 ± 300	1.10 ± 0.4

[a] Abbreviation: ALS-S, acetolactate synthase–susceptible.
[b] Values ± SE.
[c] "Partially monoecious" female ALS-S common waterhemp.
[d] Single female ALS-S common waterhemp not present with "partially moecious" female common waterhemp.

not counted as seed produced. Germination rates for the seed ranged from 5 to 46% (data not shown). Out of 3,590 potentially hybrid progeny that germinated, a total of 35 seedlings survived the herbicide treatment and later were verified as hybrids using molecular techniques (Table 2). An additional 15 plants survived the imazethapyr treatment but were not tested with Mfe1 restriction enzyme (data not shown).

On the inflorescence of one of the ALS-S common waterhemp plants, a small number of morphologically normal-looking male flowers were observed. The presence of this "partially monoecious" plant in the growth chamber may have allowed compatible common waterhemp × common waterhemp crosses to occur and may have caused the high number of seeds produced on some of the plants. Consequently, a majority of the emerged seedlings phenotypically appeared to be common waterhemp and were controlled with the imazethapyr treatment. A second possibility for the large population of these common waterhemp–type seedlings is apomictic seed production. Agamospermy apomixis is the formation of seed without the union of sperm and egg nuclei and has been reported in several agricultural crop species (Poehlman and Sleper 1995).

TABLE 2. Total numbers of seeds (±SE) and seedlings screened and the number of verified hybrids produced from 11 female ALS-S common waterhemp (*Amaranthus rudis* AMATA) plants.[a]

Female AMATA plant	Seeds screened	Seedlings screened	Verified hybrids
	Seeds/plant	Seeds/plant	Hybrids/plant
1	8,003 ± 820	989	0
2	545 ± 75	197	3
3	1,747 ± 204	246	0
4	2,930 ± 88	153	7
5	1,208 ± 77	61	2
6[b]	2,718 ± 43	725	2
7	2,320 ± 350	391	3
8	716 ± 40	182	3
9	463 ± 37	188	3
10	1,393 ± 56	442	6
11[c]	35 ± 3	16	6
Total	22,078 ± 142	3,590	35

[a] Abbreviation: ALS-S, acetolactate synthase–susceptible.
[b] "Partially monoecious" female ALS-S common waterhemp.
[c] Single female ALS-S common waterhemp not present with "partially monecious" female common waterhemp.

The ALS-S common waterhemp female (#11) that was not present in the growth chamber with the "partially monoecious" ALS-S common waterhemp female probably more accurately modeled a truly interspecific mating between common waterhemp and Palmer amaranth. This female produced fewer seeds than the other 10 females, with only 0.02% seed set (Table 1), and produced six verified hybrids from 16 viable seedlings (Table 2).

ALS molecular marker. The PCR amplification of the targeted ALS region resulted in similar-sized fragments of approximately 630 bp in the maternal ALS-S common waterhemp and paternal ALS-R Palmer amaranth (Figure 2). Comparison of the nucleotide sequences revealed a high degree of homology between the maternal and paternal lines (Figure 3). Because of the degeneracy of the genetic code, most of the differences between the ALS-S common waterhemp and ALS-R Palmer amaranth nucleotide sequences did not result in changes in the encoded amino acid (Table 3). However, a difference at nucleotide position 169 did result in a significant amino acid substitution between the ALS-S common waterhemp and ALS-R Palmer amaranth. The guanine (G) present in the ALS-S common waterhemp was substituted for a thymine (T) in the ALS-R Palmer amaranth nucleotide sequence. This substitution resulted in the encoding of a leucine amino acid in the ALS-R Palmer amaranth, whereas the ALS-S common waterhemp encoded a tryptophan at the same position. This difference in nucleotide sequences made it possible to utilize the Mfe1 restriction endonuclease to differentiate between the ALS-S common waterhemp and ALS-R Palmer amaranth forms of the ALS gene.

Digestion of the PCR fragment from ALS-R Palmer amaranth with Mfe1 resulted in two fragments of 290 and 340 bp, respectively (Figure 4). The Mfe1 digestion of the PCR fragment from ALS-S common waterhemp resulted in mul-

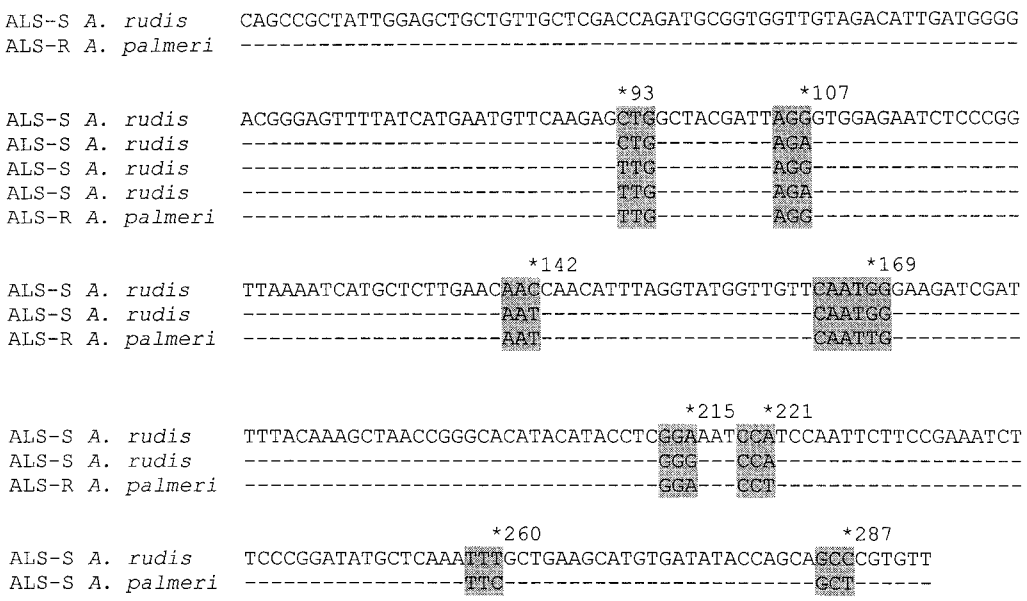

FIGURE 2. Sequence comparison of Region B of the acetolactate synthase (ALS) gene from ALS-susceptible common waterhemp (*Amaranthus rudis*) and ALS-resistant Palmer amaranth (*Amaranthus palmeri*). The Mfe1 restriction endonuclease has a recognition sequence of 5′-CAATTG-3′.

tiple banding patterns. The first banding pattern was the intact fragment of 630 bp, and the second pattern observed was a single 520-bp fragment. This could be attributed to an Mfe1 recognition sequence approximately 80 bp downstream from the forward primer. The third banding pattern observed was an apparent combination of the first two patterns. The ALS-S common waterhemp females had two visible bands: one at 630 bp and one at 550 bp (Figure 4). The 630-bp band from this third pattern was excised from the gel and transformed into a bacterial plasmid. Sequencing of the plasmid verified that the 630-bp band did not contain the Mfe1 restriction site.

Restriction digestion of the ALS PCR products of the putative interspecific hybrids verified the hybrid nature of these plants. The plants that survived the imazethapyr treatment shared digested fragments from both the female ALS-S common waterhemp and the male ALS-R Palmer amaranth, having either three or four visible fragments (Figure 4). Interspecific hybrid progeny possessed the double-fragment banding pattern associated with ALS-R Palmer amaranth in addition to having either one or two bands associated with ALS-S common waterhemp. All but three of the seedlings that survived the imazethapyr treatment possessed the hybrid-banding pattern. These individuals showed a restriction pattern identical to that of their maternal parent.

The sequence that was detected in ALS-R Palmer amaranth has been documented in *Amaranthus* species to confer broad-range resistance to the ALS-inhibiting herbicides (Woodsworth et al. 1996). A nucleotide substitution of guanine to thymine at this position in Region B of the ALS gene results in the amino acid substitution of a tryptophan in the susceptible biotype for a leucine in the resistant biotype. Other nucleotide sequence differences were observed between the ALS-S common waterhemp and ALS-R Palmer amaranth, but because of the degeneracy of the genetic code, these changes did not result in changes in the amino acid sequence (Table 3). Differences also were observed in the nucleotide sequences between the ALS-S common waterhemp females with some of the females having a codon sequence that was similar to the Palmer amaranth (Figure 2). Again, these changes did not change the amino acid composition. Similar sequence information was obtained when Foes et al. (1998) examined mechanisms of resistance

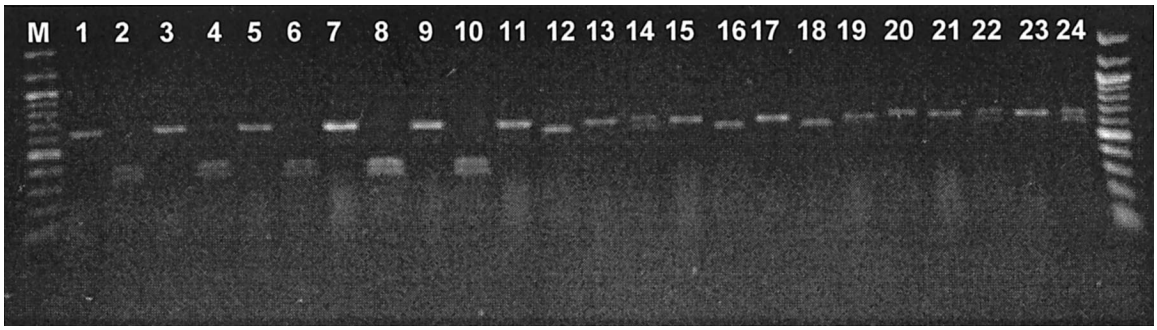

FIGURE 3. Ethidium bromide–stained gel of the restriction patterns observed from the acetolactate synthase (ALS)-resistant Palmer amaranth (*Amaranthus palmeri*) and ALS-susceptible common waterhemp (*Amaranthus rudis*). Odd-numbered lanes represent the PCR fragment prior to Mfe1 restriction digest; even-numbered lanes represent the same PCR fragment after digestion with the enzyme. (Lane M) 100-base pair reference marker, (1–10) ALS-resistant Palmer amaranth, (11–24) ALS-susceptible common waterhemp.

TABLE 3. Nucleotide polymorphisms observed betwen acetolactate synthase–susceptible (ALS-S) common waterhemp (*Amaranthus rudis* AMATA) and ALS-resistant (ALS-R) Palmer amaranth (*Amaranthus palmeri* AMAPA), the subsequently encoded amino acid, and its position.

Amino acid codon			
ALS-S AMATA	ALS-R AMAPA	Amino acid	Position[a]
C/TTG	TTG	Leucine	93
AGG/A	AGG	Arginine	107
AAC/T	AAT	Asparagine	142
TGG		Tryptophan	169
	TTG	Leucine	169
GGA/G	GGA	Glycine	215
CCA	CCT	Proline	221
TTT	TTC	Phenylalanine	260
GCC	GCT	Alanine	287

[a] Position in the nucleotide sequence relative to the forward primer.

for a common waterhemp biotype with multiple resistance to triazine and ALS herbicides.

Hybrid phenotypes. The male and female hybrid plants appeared to develop normally in regard to flower morphology. However, as the male hybrids matured, it was noted that the anthers were not dehiscing, and the male flowers appeared to be dead. Closer inspection of the inflorescences revealed a small number of presumably viable anthers dispersing pollen. This suggests that these hybrids were mostly sterile. However, Wetzel et al. (1999) demonstrated that interspecific hybridization between common waterhemp and Palmer amaranth could produce a small number of progeny and that these progeny could be backcrossed successfully to a parental line.

One of the hybrids exhibited the "partially monoecious" flowering characteristics similar to the maternal ALS-S common waterhemp. However, instead of only a few male flowers present among a predominately female inflorescence, one entire male inflorescence was found on a plant that was predominately female. This suggests that the characteristic is a heritable trait that possibly could be used in future studies of sex determination in *Amaranthus.*

For the most part, hybrid progeny did not appear to be phenotypically intermediate between the common waterhemp or Palmer amaranth parental lines. The verified hybrid progeny typically resembled Palmer amaranth with a few exceptions. A common characteristic in Palmer amaranth is a leaf chevron or V-mark. This leaf characteristic was present on many of the verified hybrids that resembled Palmer amaranth but also was present on one putative hybrid that more closely resembled common waterhemp. Kulakow et al. (1985) determined that two dominant genes probably control this trait in cultivated amaranths. The presence of this mark in a waterhemp-like plant hybrid further verified its hybrid nature.

Similarly, another trait that is typical of cultivated species of *Amaranthus* is a drooping panicle that usually is associated with *Amaranthus caudatus* L. The main stems of these plants are erect, but the inflorescences tend to droop as if heavily laden with seed. This characteristic was expressed strongly

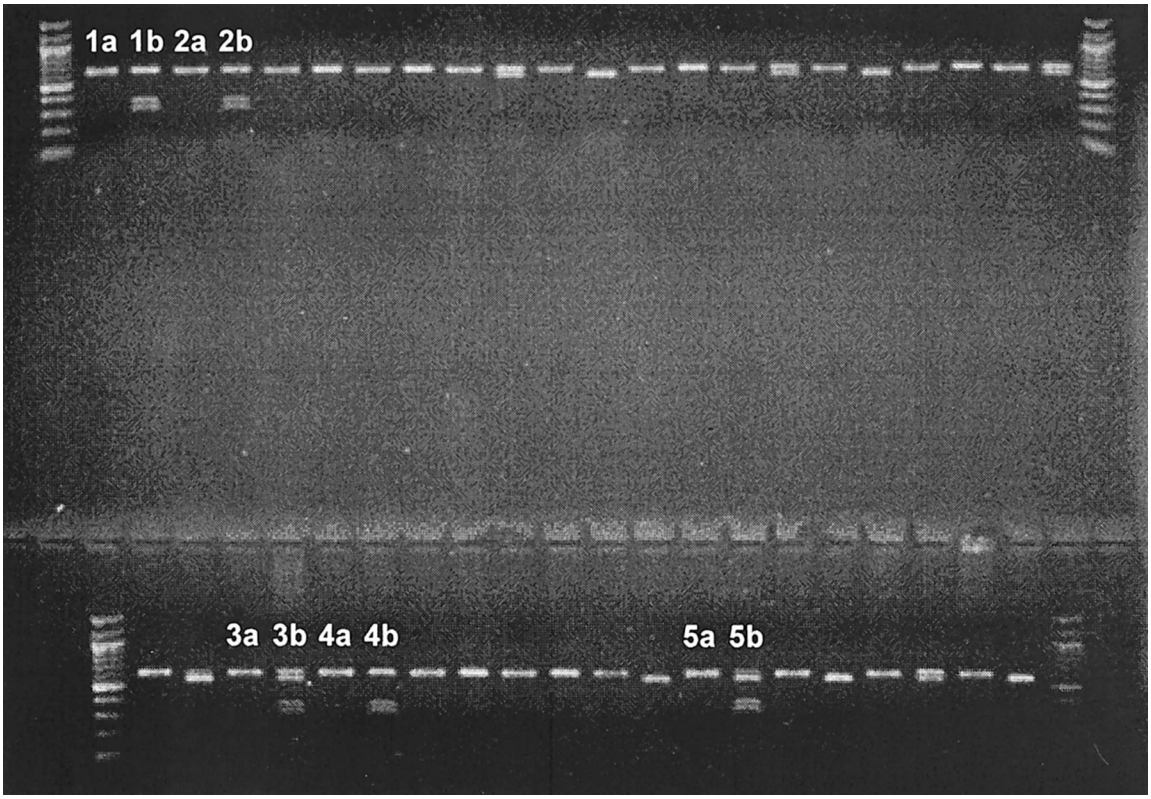

FIGURE 4. Ethidium bromide–stained gel of the restriction patterns observed from the random screening of hybrid seed prior to herbicide treatment. Marked lanes 1 to 5 are paired for each individual and represent those individuals that survived imazethapyr treatment. Unmarked lanes represent individuals that did not survive the imazethapyr treatment. Lanes labeled with the letter "a" and "b" are the undigested and digested PCR fragment of the same individual, respectively.

in one verified male hybrid and was expressed partially in a second male hybrid. This drooping panicle characteristic is controlled by two dominant genes with the possibility of additional modifying genes in cultivated *Amaranthus* species (Kulakow 1987).

Some of the hybrids showed an extreme lack of internode elongation and wrinkled and deformed leaves. These plants were almost void of lateral branching and developed a shrub-like appearance. Other plants that did not lack internode elongation also expressed a wrinkling of the leaves. Pal and Khoshoo (1972) performed interspecific crosses between the grain species *A. caudatus* and *Amaranthus hypochondriacus* L. and reported what they called virus-like symptoms of wrinkled deformed leaves with tumorous stems and roots in the interspecific hybrids.

The appearance of these traits normally not seen in weedy species might be due to the combining and reorganization of the hybrid genomes. Amplified fragment length polymorphism (AFLP) studies conducted by Wetzel et al. (1999) showed a high number of nonparental restriction fragments present in the hybrid progeny, suggesting a restructuring of the genome.

These results suggest that formation of interspecific hybrids between common waterhemp and Palmer amaranth probably does not occur at a very high frequency in the wild. In this study, we were able to produce 35 interspecific hybrids from 22,000 seeds that were planted. However, this study did not take into account factors such as natural pollen load or the degree of synchronization in flowering times of these two species. In wild populations found in Kansas, Palmer amaranth emerges before common waterhemp. Although initiation of flowering in the wild has not been determined for these species, their flowering times likely are not completely synchronized, but may overlap. These factors probably reduce the frequency of natural interspecific hybridization. Nevertheless, the possibility of herbicide resistance developing from a single base pair substitution, the obligate out-crossing nature of the dioecious *Amaranthus* species, and the results reported here indicate that hybridization could contribute to the rapid spread of ALS resistance within and between *Amaranthus* species.

Sources of Materials

[1] Jiffy Mix. Jiffy Products of America, Inc., 951 Swanson Drive, Batavia, IL 60510.

[2] Miracle-Gro® soluble fertilizer. Scotts Miracle-Gro Products Inc., Consumer Products Division, 800 Port Washington Boulevard, Port Washington, NY 11050.

[3] Pursuit 2 AS. American Cyanimid Company, Agricultural Products Research Division, P.O. Box 400, Princeton, NJ 08543.

[4] Kenetic. Setre Chemical Co., 6075 Poplar, Suite 500, Memphis, TN 38137.

[5] DeVries Manufacturing, RR 1 Box 184, Hollendale, MN 56045.

[6] Spraying Systems Co., P.O. Box 7900, Wheaton, IL 60189.

[7] Beckman DU 640 Spectrophotometer. Beckman Coulter, Inc., Bioindustrial Business Unit, 4300 N. Harbor Boulevard, P.O. Box 3100, Fullerton, CA 92834.

[8] IDT Technologies, 2900 Westown Parkway, West Des Moines, IA, 50226

[9] Biolase DNA Polymerase. Bioline USA Inc., Reno, NV 89502.

[10] PTC-100 Programmable Thermal Controller. MJ Research Inc., 590 Lincoln Street, Waltham, MA 02451.

[11] SeaKem® LE Agarose. FMC BioProducts, 191 Thomaston Street, Rockland, ME 04841.

[12] Mass ladder (100-bp). New England Biolabs, 32 Tozer Road, Beverly, MA 01915.

[13] Millipore Ultrafree®-DA. Millipore Corp., 80 Ashby Road, Bedford, MA 01730.

[14] ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit. PE Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404.

[15] KSU-USDA Sequencing and Genotyping Facility, 4006 Throckmorton Hall, Kansas State University, Manhattan, KS 66506.

[16] Mfe1 restriction enzyme. New England Biolabs, 32 Tozer Road, Beverly, MA 0915.

[17] pGEM®-T Easy Vector Systems Kit. Promega Corp., 2800 Woods Hollow Road, Madison, WI 53711.

[18] JM109 High Efficiency Competent Cells. Promega Corp., 2800 Woods Hollow Road, Madison, WI 53711.

[19] L-Broth Bacterial Growth Media. BIO101 INC., 1070 Joshua Way, Vista, CA 92083.

[20] QIAprep® Miniprep. QIAGEN Inc., 28159 Stanford, Valencia, CA 91355.

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